



TITLE: Detection of benzimidazole carbamates and amino metabolites in liver by surface plasmon resonance-biosensor

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1 **Detection of benzimidazole carbamates and amino metabolites in liver by surface**  
2 **plasmon resonance-biosensor**

3  
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21

22 **Abstract**

23 Two surface plasmon resonance (SPR) biosensor screening assays were developed and  
24 validated to detect 11 benzimidazole carbamate (BZT) and four amino-benzimidazole  
25 veterinary drug residues in liver tissue. The assays used polyclonal antibodies, raised in  
26 sheep, to detect BZTs and amino-benzimidazoles. A modified Quick, Easy, Cheap,  
27 Effective, Rugged and Safe (QuEChERS) extraction method was developed to isolate  
28 benzimidazole carbamate residues. Liver samples were extracted using an acetonitrile  
29 extraction method. BZTs were purified by dispersive solid phase extraction (d-SPE) using  
30 C<sub>18</sub> sorbent. Residues of amino-benzimidazoles were effectively cleaned-up using a simple  
31 cyclohexane defatting step. The assays were validated in accordance with the performance  
32 criteria described in 2002/657/EC. The BZT assay limit of detection was calculated to be  
33 32 µg kg<sup>-1</sup>, the detection capability (CC<sub>β</sub>) was determined to be 50 µg kg<sup>-1</sup> and the mean

34 recovery of analytes was in the range 77-132%. The amino-benzimidazole assay limit of  
35 detection was determined to be  $41 \mu\text{g kg}^{-1}$ , the  $\text{CC}\beta$  was determined to be  $75 \mu\text{g kg}^{-1}$  and  
36 analyte recovery was in the range 103-116%. Biosensor assay performance was tested by  
37 analysing liver tissue from animals treated with benzimidazole drugs and comparing the  
38 results with an ultra high performance liquid chromatography tandem mass spectrometry  
39 (UHPLC-MS/MS) confirmatory method. All non-compliant samples were identified using  
40 the biosensor assays.

41

42 **Keywords:** SPR biosensor, Benzimidazoles, Liver, Screening Assay

43

44

## 45 **1. Introduction**

46 Benzimidazoles are anthelmintic agents with broad spectrum activity against nematodes,  
47 cestodes and trematodes. They are widely used for treatment of food producing animals in  
48 the European Union (EU). Many benzimidazole drugs have been proven to be safe when  
49 product label claims are followed. However, some drugs have shown teratogenic  
50 properties and congenital malformations have been reported in gestating ewes after the  
51 administration of albendazole and oxfendazole ABZ and OFZ [1]. Hence concern has been  
52 raised that high levels of residues may affect developing embryos in pregnant women.  
53 Maximum residue limits (MRLs) have been established in the EU for benzimidazole  
54 residues in edible tissues to protect public health under Commission Regulation  
55 2010/37/EC. In addition, annual surveillance programmes are carried out in member states

56 under Council Directive 96/23/EC. Results from this surveillance highlight the need for  
57 continued monitoring of benzimidazole residues due to sporadic incidences of non-  
58 compliant benzimidazole residues in milk and meat [2].

59

60 Several assays have been reported for benzimidazoles in liver because residues are known  
61 to accumulate in this organ making it an ideal target tissue for residue surveillance  
62 purposes. Marti et al. developed a HPLC-UV method to detect eight benzimidazole  
63 residues in liver tissue using an acetonitrile extraction followed by purification with  
64 multiple liquid-liquid partitioning (LLP) and solid phase extraction (SPE) steps [3].  
65 Wilson et al. subsequently developed a simpler method based on ethyl acetate extraction  
66 coupled with purification by LLP (acidified ethanol versus hexane) and a further C<sub>2</sub> SPE  
67 clean-up step prior to HPLC-UV analysis [4]. This method has been used in many  
68 laboratories, but analyte coverage has been limited both by inadequate HPLC resolution  
69 and the poor availability of analytical standards [5]. The method has been extended to 12  
70 benzimidazole residues but the throughput of the assay has been limited in our laboratory  
71 to 38 samples and associated controls per week for a single analyst [6]. Other groups have  
72 increased the analytical scope and throughput of methods using liquid chromatography  
73 coupled to mass spectrometry [7]. However, this technology requires a significant amount  
74 of consumables, extensive maintenance for effective continued operation and experienced  
75 operators to process results. Ideally a method should include a wide range of  
76 benzimidazole marker residues as listed in Table 1. The inclusion of metabolites is  
77 important because they can be more toxic and persist longer than the parent drug [8-10].

78

79 Enzyme linked immunosorbent assays (ELISAs) have been developed by some groups for  
80 detecting benzimidazole residues as easier and low cost alternatives to chemical assays  
81 [11]. Immunoassays can also offer similar selectivity and sensitivity to that of LC-MS/MS.  
82 However, they are slow because of the multiple washing and incubation steps required and  
83 have proved difficult to automate in food analysis [12]. In addition, ELISAs with good  
84 repeatability can be difficult to develop and results are frequently non-quantitative and  
85 susceptible to matrix effects. Alternatively, several surface plasmon resonance (SPR)  
86 optical biosensor assays have been developed for detecting low levels of contaminant  
87 residues in food [13-15]. SPR is advantageous because labelling of antibodies or enzymes  
88 is not required. Furthermore, assay performance is frequently enhanced through  
89 automation and real time analysis. Crooks et al. highlighted the advantage of SPR  
90 biosensor over ELISA for analysing sulphonamide residues in 2081 pig bile samples over  
91 an eight month period [16]. False positive rates of 0.14 to 0.34% and 1.44 to 1.54% were  
92 observed for SPR-biosensor and ELISA, respectively. No false negative results were  
93 observed using SPR biosensor, while ELISA false negative rates were 0.14 to 0.24%.

94 The cost of SPR biosensors is higher than ELISA but savings can be seen through  
95 automation, which reduces labour costs and improves reliability of results. A further  
96 advantage of the technique is the ease of assay transfer between laboratories through  
97 reduction of operator effects through the elimination of plate washing and incubation steps.  
98 In addition, clear advantages of SPR biosensor assay can be seen over traditional HPLC  
99 based detection systems, which are frequently dedicated to specific assays, require  
100 laborious sample preparation and take longer to set-up prior to analysis. A typical SPR  
101 biosensor system can handle as many as five different assays in a single week because of

102 the speed of changeover. In addition, recent advances in instrumentation have highlighted  
103 the ability to multiplex assays and improve sample throughput [17].

104

105 Recently a biosensor screening assay for benzimidazole carbamate residues in milk was  
106 developed using a modified QuEChERS extraction [18]. Due to limitations in the cross-  
107 reactivity of the antibody this assay could not detect a number of key metabolites  
108 possessing amino functional groups. The assay was found to be suitable for screening  
109 residues in incurred milk samples to below the MRL but extension to amino metabolites is  
110 desirable to provide more quantitative results. The aim of this study was to develop and  
111 validate multi-residue SPR biosensor assays to screen liver tissue samples for 11  
112 benzimidazoles and four amino-benzimidazole residues. The suitability of the assays to  
113 detect residues in liver tissue was verified through application of each assay to samples that  
114 were previously shown to contain benzimidazole residues by UHPLC-MS/MS. The  
115 method was validated according to 2002/657/EC [19].

116

117

## 118 **2. Materials and methods**

119

### 120 *2.1 Chemicals, reagents and apparatus*

121 CM5 sensor chips (research grade), 96 well polystyrene microplates, NHS (100 mM N-  
122 hydroxysuccinimide in water), EDC (400 mM 1-ethyl-3-(3-dimethylaminopropyl)-

123 carbodiimide hydrochloride in water), 1 M ethanolamine and HBS-EP buffer (10 mM  
124 H EPES pH 7.4 with 0.05 M NaCl, 3.4 mM EDTA) and 0.005% (v/v) P20 were all obtained  
125 from GE Healthcare (Uppsala, Sweden). Ultra-pure water (18.2 MΩ) was generated in-  
126 house using a Millipore water purification system (Cork, Ireland). Sodium hydroxide  
127 (NaOH), pesticide grade acetonitrile (MeCN), pesticide grade dimethylsulphoxide  
128 (DMSO), pesticide grade ethyl acetate, cyclohexane and methanol were supplied by  
129 BDH/VWR international Ltd. (Poole, England, UK). Ethylenediamine (99%, v/v),  
130 dimethylformamide (DMF), albendazole (ABZ), mebendazole (MBZ) and fenbendazole  
131 (FBZ) were supplied by Sigma Aldrich (Steinheim, Germany). Oxibendazole (OXI),  
132 fenbendazole-sulphoxide (FBZ-SO) and flubendazole (FLU) were purchased from QMX  
133 laboratories (Thaxted, UK). Amino-flubendazole (FLU-NH<sub>2</sub>), amino-mebendazole (MBZ-  
134 NH<sub>2</sub>), hydroxy-mebendazole (MBZ-OH), and hydroxy-flubendazole (FLU-OH) were  
135 received as a gift from Janssen pharmaceuticals (Belgium). Albendazole-2-amino-  
136 sulphone (ABZ-NH<sub>2</sub>-SO<sub>2</sub>), albendazole sulphone (ABZ-SO), albendazole sulphoxide  
137 (ABZ-SO), fenbendazole sulphone (FBZ-SO<sub>2</sub>) and amino-oxibendazole (OXI-NH<sub>2</sub>) were  
138 purchased from Witega laboratories (Berlin, Germany). Polypropylene centrifuge tubes  
139 with screw caps (50 mL) containing 4 g magnesium sulphate (MgSO<sub>4</sub>) and 1 g NaCl were  
140 supplied by United Chemical Technologies (Bristol, PA, USA). Polypropylene tubes (50  
141 mL) containing 1.5 g magnesium sulphate (MgSO<sub>4</sub>) and 0.5 g C<sub>18</sub> were purchased from  
142 Biotage (Uppsala, Sweden). The amino-albendazole hapten (Lot no. LK515) stored at -  
143 20°C was received from Radox Life Sciences (Antrim, Northern Ireland). Whatman®  
144 syringe filter units (polytetrafluoroethylene (PTFE), 0.2 μm) were purchased from Fisher  
145 Scientific (Dublin, Ireland). Primary standard stock solutions (1 mg mL<sup>-1</sup>) for each  
146 benzimidazole were prepared in DMSO. Working standard solutions were then prepared at

147 40  $\mu\text{g mL}^{-1}$  by diluting the primary stock in methanol. A FASTH 21 homogenisation unit  
148 and sample homogenisation tubes were supplied by Syntec Scientific (Dublin, Ireland), a  
149 Mistral 3000i centrifuge (MSE, London, UK), an Elma Transsonic T780/H ultrasonic bath  
150 (Bedford, UK) and a Turbovap LV evaporator (Caliper Life Sciences, Runcorn, UK) were  
151 used during sample preparation.

152

## 153 2.2 *Liver samples*

154

### 155 2.2.1 *Negative control samples*

156 Ovine liver samples found to be free of benzimidazole residues by UHPLC-MS/MS, with a  
157 limit of detection (LOD) of  $<1 \mu\text{g kg}^{-1}$ , were used as negative controls.

### 158 2.2.2 *Incurred liver samples*

159 The suitability of the assay to detect residues was evaluated through application to fortified  
160 and naturally positive samples. Liver tissue samples purchased from a supermarket  
161 (samples 1-7) were tested to establish the performance of the assay when low levels of  
162 benzimidazole residues are present. To prepare incurred samples, three 16-month old  
163 steers were dosed orally with mebendazole (sample 8), fenbendazole (sample 9) and  
164 albendazole (sample 10) at 15, 7.5 and 5  $\text{mg kg}^{-1}$  body weight, respectively. The animals  
165 were humanely euthanized after 24 h and the livers were collected and stored at  $-20^\circ\text{C}$  until  
166 analysis. The UHPLC-MS/MS sample preparation, detection conditions and calibration  
167 method used in this work were outlined in recent work reported by Kinsella et al. [20].

168

## 169 2.3 SPR-Biosensor Assays

### 170 2.3.1 Sample preparation

171 A modified QuEChERS extraction method was used to isolate benzimidazole carbamate  
172 residues from liver tissue. Finely chopped liver (2 g) was homogenised in a slurry  
173 containing MeCN:MgSO<sub>4</sub>:NaCl (12:4:1, v/w/w), homogenised (30 sec in a multi-  
174 homogenisation unit) and centrifuged (3,000 ×g, 10 min, -5°C). The supernatant was  
175 transferred to a tube containing C<sub>18</sub> sorbent (500 mg) and MgSO<sub>4</sub> (1.5 g). The tubes were  
176 subsequently shaken (1 min) and centrifuged (3500 ×g, 10 min, -5°C). The MeCN layer (6  
177 mL) was transferred to polypropylene tubes and DMSO (500 µL) was added. The MeCN  
178 was evaporated under nitrogen at 50°C using a Turbovap LV (Caliper Life Sciences,  
179 Runcorn, UK). The DMSO extracts were vortexed (2 min) and sonicated (10 min).

180 Amino-benzimidazole residues were extracted using the same procedure as for the  
181 carbamate metabolites but did not undergo C<sub>18</sub> clean-up. Instead, DMSO extracts were  
182 defatted with cyclohexane (2 × 2 mL aliquots), and the cyclohexane layer was removed by  
183 aspiration. DMSO sample extracts were vortexed (2 min) and sonicated (10 min).

184

### 185 2.3.2 SPR-biosensor chip preparation

186 The preparation of the biosensor chip for benzimidazole carbamate was described in  
187 previous work by this research group [18].

188 A new CM5 biosensor chip was prepared for amino-benzimidazoles. Firstly the chip was  
189 left to equilibrate to room temperature (20 min). HBS-EP buffer (50  $\mu$ L) was added to  
190 each chip surface and incubated (10 min). The buffer was removed and 50 mM NHS:200  
191 mM EDC (1:1, v/v, 40  $\mu$ L) was added to the chip and incubated (20 min, room  
192 temperature) to activate the surface. This solution was removed from the surface. An  
193 amine surface was prepared by adding 1 M ethylenediamine (50  $\mu$ L) to the surface (1 h,  
194 room temperature). The solution was removed using lint-free tissue paper. A carboxy-  
195 amino-albendazole derivative (2.5 mg) was dissolved in DMF (1 mL), vortexed (2 min)  
196 and sonicated (15 min). EDC (1.825 mg) and NHS (1.25 mg) were added to this solution  
197 and incubated at room temperature (3 h) to activate the carboxyl groups of the amino-  
198 benzimidazole derivative to form o-acylisourea intermediates with a COOH function. The  
199 remaining unreacted groups on the chip surface were deactivated by addition of 1 M  
200 ethanolamine-HCl (50  $\mu$ L) and allowed to react (20 min). Following immobilization, the  
201 chip was washed five times with HBS-EP buffer and dried under a nitrogen stream. The  
202 amino-albendazole immobilized chip was stored in a Sarstedt® tube containing silica  
203 crystals (4°C) when not in use.

204

### 205 2.3.3 SPR-biosensor analytical cycle

206 The optical biosensor used was a Biacore Q (GE Healthcare, Uppsala Sweden) with  
207 Biacore®Q control software version 3.0. BIAevaluation software 3.0.1 was used for data  
208 handling. Studies were conducted at 25°C and all samples and calibrants were analysed in  
209 duplicate.

210 Polyclonal antibody, raised in sheep against 5(6)-[(carboxypentyl)-thio]-2-

211 benzimidazolecarbamate derivative (CMB) coupled to human serum albumin (HSA), was  
212 received from the Veterinary Sciences Division, Agri-Food and Biosciences Institute,  
213 Belfast, Northern Ireland and was used for the benzimidazole carbamate assay. An  
214 antibody dilution of 1/1000 (v/v), was found to give satisfactory results under assay  
215 conditions. DMSO extracts were transferred to 96 well microplates and mixed (1:9, v/v)  
216 with antibody and passed over the immobilised surface at  $10 \mu\text{L min}^{-1}$  (2 min).  
217 Regeneration of the chip was carried out by sequential injection of 25 mM HCl (15  $\mu\text{L}$ )  
218 followed by 180 mM NaOH (20  $\mu\text{L}$ ) across the chip at  $25 \mu\text{L min}^{-1}$ .

219 Polyclonal sheep antibody raised against amino-albendazole coupled to bovine  
220 thyroglobulin (BTG) was from Randox Laboratories (Crumlin, Northern Ireland) and was  
221 used for amino-benzimidazole detection. The Ig fraction (2.4 mg mL<sup>-1</sup> in phosphate-  
222 buffered saline containing 0.09% sodium azide) was diluted 1/400 (v/v), to give  
223 satisfactory results under assay conditions. DMSO sample extracts were diluted in HBS-  
224 EP buffer (1:4, v/v), added to a 96 well microplate and mixed with (1:4, v/v) antibody and  
225 passed over the chip surface at  $10 \mu\text{L min}^{-1}$  (3 min). Regeneration of the chip was carried  
226 out by sequential injection of 25 mM HCl (15  $\mu\text{L}$ ) and 170 mM NaOH (20  $\mu\text{L}$ ) at  $25 \mu\text{L}$   
227  $\text{min}^{-1}$ . The binding of the antibody to the chip surface was measured as the change in SPR  
228 signal between two report points, 10 sec before and 30 sec after each injection. A  
229 competitive immunoassay format was used to detect inhibition of antibody binding to the  
230 chip surface. The SPR signal was expressed in arbitrary resonance units (RU).

231

#### 232 2.4 Calibration

233 Benzimidazole residue-free liver samples were fortified with albendazole-sulphone (ABZ-

234 SO<sub>2</sub>) at levels of 0, 50, 100, 250, 500 and 1000 µg kg<sup>-1</sup> to prepare an extract calibration  
235 curve for the benzimidazole carbamate assay. Similarly samples were fortified with  
236 albendazole-amino-sulphone (ABZ-NH<sub>2</sub>-SO<sub>2</sub>) at levels of 0, 25, 50, 75, 125, 250 and 500  
237 µg kg<sup>-1</sup> to prepare an extract calibration curve for the amino-benzimidazole assay.  
238 BIAevaluation software was used to construct inhibition assay standard curves based on a  
239 4-parameter fit.

240

#### 241 2.5 Method validation

242 A qualitative approach was used to determine the performance factor CC<sub>β</sub> (detection  
243 capability) as described in 2002/657/EC criteria [19]. Firstly, the limit of detection (LOD)  
244 of the assay was determined by measuring the mean response for 20 different negative  
245 ovine liver tissue samples and subtracting three standard deviations. CC<sub>β</sub> is the  
246 concentration at which a substance can be identified as positive (>LOD) with a statistical  
247 certainty of (1-β), where β = 5%. In order to determine CC<sub>β</sub> for each assay, samples (n =  
248 20 for each analyte) were spiked at a concentration above the LOD. If 19 of the 20  
249 fortified samples were identified as positive, CC<sub>β</sub> was to be determined to be equal to the  
250 fortification level (5% probability of a false negative result). If 20 samples were identified  
251 as positive, CC<sub>β</sub> was determined to be less than the fortification level and if ≤18 samples  
252 were identified as positive, CC<sub>β</sub> was determined to be greater than the fortification level.  
253 Liver samples were fortified at arbitrary concentrations above the LOD of each assay and  
254 through trial and error CC<sub>β</sub> levels were determined. Assay repeatability was evaluated by  
255 extracting and analysing ovine liver fortified with each analyte on five separate days.

256

257

### 258 **3. Results and discussion**

259

#### 260 *3.1 Development of sample preparation procedures*

261 Several sample preparation procedures have been developed for the isolation of  
262 benzimidazole residues from liver tissue based on liquid-liquid extraction with a water  
263 immiscible solvent such as ethyl acetate. An ethyl acetate extraction procedure (extraction  
264 procedure I) based on the method reported by Dowling et al. [9] was evaluated for the  
265 isolation of benzimidazole carbamates from liver tissue. The automated SPE clean-up step  
266 was omitted because it was considered unsuitable for a rapid method. After centrifugation,  
267 the ethyl acetate supernatant was reduced to dryness under nitrogen (50°C) and  
268 resuspended in MeOH:water (50:50, v/v). This extract was diluted (1/20, v/v) in HBS-EP  
269 buffer prior to biosensor analysis. Extracted matrix calibration curves prepared over the  
270 range 0 to 2000  $\mu\text{g kg}^{-1}$  (ABZ-SO equivalents) showed significant lower sensitivity ( $\text{IC}_{50} =$   
271  $770 \mu\text{g kg}^{-1}$ ) when compared to buffer curves ( $\text{IC}_{50} = 88 \mu\text{g kg}^{-1}$ ) (Fig. 1). Losses in  
272 recovery were due to adsorption of analytes onto filter paper containing sodium sulphate.  
273 Subsequently the sample preparation procedure was modified by reducing the weights of  
274 sample and sodium sulphate (extraction procedure II) but this resulted in only slight  
275 improvements in sensitivity ( $\text{IC}_{50} = 625 \mu\text{g kg}^{-1}$ ).

276

277 An alternative MeCN extraction was next evaluated for isolating benzimidazoles from liver  
278 tissue [6]. MeCN is an attractive solvent for isolating benzimidazole residues from

279 biological samples without pH adjustment, extracts a lower quantity of fat and precipitates  
280 protein. Simple liquid-liquid partitioning steps were employed based on cyclohexane and a  
281 saturated aqueous NaCl wash to remove non-polar and polar matrix components,  
282 respectively. This sample preparation approach resulted in a significant improvement in  
283 sensitivity. The calibration curve in liver matrix showed an  $IC_{50}$  of  $89 \mu\text{g kg}^{-1}$  (extraction  
284 procedure III), not significantly different from the  $IC_{50}$  ( $88 \mu\text{g kg}^{-1}$ ) in buffer. However, the  
285 sensitivity required for the recovery for ABZ and FBZ residues was unsatisfactory at  
286  $<40\%$ .

287 In earlier work by the present research group, a QuEChERS sample preparation procedure  
288 had been successfully applied to the analysis of 11 benzimidazole residues in milk samples.  
289 However, we evaluated an alternative clean-up procedure for liver tissue analysis because  
290 of the lower sensitivity required. A QuEChERS sample preparation procedure was applied  
291 to fortified ovine liver extracts, and the calibration curve showed comparable sensitivity  
292 ( $IC_{50} = 86 \mu\text{g kg}^{-1}$ ) to MeCN and buffer curves. In addition, recoveries of ABZ and FBZ  
293 were acceptable, and the assay proceeded to validation. Subsequently, a new antibody  
294 became available that showed specificity towards amino-benzimidazole metabolites.  
295 Initially, the d-SPE procedure described in section 2.3.1 was used for amino-benzimidazole  
296 extraction but showed consistently low recovery of  $<50\%$  for FLU-NH<sub>2</sub>, MHZ-NH<sub>2</sub> and  
297 OXI-NH<sub>2</sub> residues. Spiking experiments verified that this loss occurred at the clean-up  
298 stage. Alternative clean-up methods were investigated using different brands of C<sub>18</sub>  
299 sorbents, high speed centrifugation ( $18000 \times g$ ), and washing with cyclohexane. Liquid-  
300 liquid partitioning with cyclohexane showed the highest recovery levels for all amino-  
301 metabolites and was selected for further validation.

302

303 *3.2 Antibody inhibition studies*

304 The cross-reactivity of the benzimidazole carbamate (S48) polyclonal antibody was  
305 determined in previous work by analysing inhibition curves obtained for each of 11  
306 analytes in buffer by the SPR-biosensor assay [18]. The cross-reactivity of the S48  
307 antibody towards 11 benzimidazole carbamates was determined by analysing inhibition  
308 curves in ovine liver tissue (0-1000  $\mu\text{g kg}^{-1}$ ) using the QuEChERS method.  $\text{IC}_{50}$  values in  
309 matrix ranged from 78 to 95  $\mu\text{g kg}^{-1}$  for FBZ-SO and FBZ, respectively, and cross-  
310 reactivities at 50% inhibition ( $\text{CR}_{50}$ ) were 110 and 91% respectively (Table 2). Matrix  
311 calibration curves for 11 benzimidazole carbamates are shown in Fig. 2.

312 The cross-reactivity of the anti-amino-benzimidazole polyclonal antibody (PAS 9869) was  
313 determined by analysing inhibition curves with analyte concentrations from 0 - 125  $\text{ng mL}^{-1}$   
314 prepared in HBS-EP buffer and from 0 - 500  $\mu\text{g kg}^{-1}$  in ovine liver tissue. In buffer the  
315 antibody showed significant cross-reactivity with four amino-benzimidazoles (80 to 125%)  
316 in the following order of affinity OXI-NH<sub>2</sub>>MBZ-NH<sub>2</sub>>ABZ-NH<sub>2</sub>-SO<sub>2</sub>>FLU-NH<sub>2</sub> and  
317 analyte  $\text{IC}_{50}$  values were typically less than 7.1  $\text{ng mL}^{-1}$  (Table 2).  $\text{IC}_{50}$  values in matrix  
318 ranged from 35 to 55  $\mu\text{g kg}^{-1}$  for the four amino analytes. Matrix calibration curves for  
319 four amino-benzimidazoles are shown in Fig. 3.

320

321 *3.3 Method Validation*

322

323 *3.3.1. Benzimidazole carbamate biosensor assay*

324 The dynamic range of the assay was found to be from  $7 \mu\text{g kg}^{-1}$  ( $\text{IC}_{10}$ ) to  $340 \mu\text{g kg}^{-1}$  ( $\text{IC}_{90}$ )  
325 and the  $\text{IC}_{50}$  was calculated to be  $86 \mu\text{g kg}^{-1}$ . The LOD was determined to be  $32 \mu\text{g kg}^{-1}$  by  
326 measuring the mean response of 20 representative blank ovine liver samples (459 RU) and  
327 subtracting three standard deviations ( $3 \times 24$  RU). To determine the  $\text{CC}\beta$  a concentration  
328 of  $50 \mu\text{g kg}^{-1}$  was selected; this is equivalent to one quarter of the concentration of the  
329 analyte with the lowest MRL. The results for the determination of  $\text{CC}\beta$  for each analyte  
330 are shown in Table 3. The  $\text{CC}\beta$  for ten of the analytes was found to be less than  $50 \mu\text{g kg}^{-1}$ .  
331 The  $\text{CC}\beta$  for MBZ-OH was found to be equal to  $50 \mu\text{g kg}^{-1}$  where one sample was not  
332 identified as positive; the false negative sample gave a measured result of  $32 \mu\text{g kg}^{-1}$ .  
333 However the method satisfies the false negative rate (5%) as required by 2002/657/EC. The  
334 repeatability of the assay was evaluated by analysing fortified ovine liver samples ( $100 \mu\text{g}$   
335  $\text{kg}^{-1}$ ) with the 11 analytes on five separate days (Table 3). Results showed acceptable  
336 recovery (77-132%) and inter-assay coefficients of variation (11-17%) for the purposes of a  
337 screening method. Calibration curves for each day are shown in Fig. 4(A).

338

### 339 3.3.2 Amino benzimidazole assay

340 The dynamic range of the assay was found to be from 22 ( $\text{IC}_{10}$ ) to  $238 \mu\text{g kg}^{-1}$  ( $\text{IC}_{90}$ ) and  
341 the  $\text{IC}_{50}$  was  $44 \mu\text{g kg}^{-1}$ . The LOD of the assay using was determined to be  $41 \mu\text{g kg}^{-1}$  by  
342 measuring the mean response of 20 representative blank ovine liver samples (236 RU) and  
343 subtracting three standard deviations ( $3 \times 21$  RU).

344 The  $\text{CC}\beta$  of the assay was determined by fortifying 20 representative blank ovine liver  
345 samples at  $75 \mu\text{g kg}^{-1}$  with four different amino-benzimidazoles. The  $\text{CC}\beta$  for three of the  
346 four amino analytes was found to be  $<75 \mu\text{g kg}^{-1}$  because all 20 fortified samples showed

347 responses above the LOD (Table 3). The  $CC\beta$  for FLU-NH<sub>2</sub> was equal to 75  $\mu\text{g kg}^{-1}$  as one  
348 of the samples gave a measured result of 40  $\mu\text{g kg}^{-1}$  and was deemed negative. The  
349 repeatability of the assay was evaluated by analysing ovine liver samples fortified (125  $\mu\text{g}$   
350  $\text{kg}^{-1}$ ) with four analytes on five separate days. Results showed acceptable recovery (103-  
351 116%) and inter-assay coefficients of variation (8-16%) for the purposes of a screening  
352 method (Table 3). Calibration curves for each day are shown in Fig. 4(B).

353

#### 354 *3.4 Application of SPR assay to incurred liver tissue*

355 The suitability of the SPR biosensor assays was evaluated by analysing three liver tissue  
356 samples from bovine animals treated with albendazole, fenbendazole and mebendazole  
357 products and seven supermarket samples found to contain benzimidazole residues. The  
358 samples were independently analysed by two different analysts using the SPR-biosensor  
359 and UHPLC-MS/MS methods. Seven of the nine samples were found to contain  
360 benzimidazole residues at concentrations above the LOD, which was 32 and 41  $\mu\text{g kg}^{-1}$  for  
361 the benzimidazole carbamate and amino-benzimidazole SPR-biosensor assays, respectively  
362 (Table 4). Samples one to six were determined to be compliant for benzimidazole residues  
363 by both the biosensor assay and UHPLC-MS/MS. Two of these samples (five and six)  
364 screened above  $CC\beta$  by the benzimidazole carbamate SPR-biosensor assay, which indicate  
365 that they should be sent for confirmatory analysis. A total of four samples (7 to 10) were  
366 confirmed to be non-compliant by UHPLC-MS/MS. Three samples (7, 9 and 10) contained  
367 residues above their respective MRLs. The remaining sample, number 8, was categorised  
368 as non-compliant because it contained MBZ residues, which are not allowed in bovine  
369 animals. The benefits of analysing samples using the amino-benzimidazole biosensor

370 assay can be seen from the results for samples 8 and 10, which gave a screening response  
371  $>CC\beta$ . UHPLC-MS/MS confirmed these samples to contain MBZ-NH<sub>2</sub> and ABZ-NH<sub>2</sub>-  
372 SO<sub>2</sub> residues at 244 and 228  $\mu\text{g kg}^{-1}$  respectively.

373 One notable aspect of this work was that no amino-benzimidazole response was detected in  
374 samples confirmed positive for FBZ residues, particularly samples 7 and 9, which were  
375 determined by UHPLC-MS/MS to contain FBZ marker residues at concentrations above  
376 1000  $\mu\text{g kg}^{-1}$ .

377

378

#### 379 4. Conclusions

380 The SPR-biosensor assays presented in this work are suitable for use as rapid screening  
381 methods for the detection of 11 benzimidazole carbamate residues and four amino-  
382 benzimidazole residues in ovine liver tissue. Both assays were validated according to  
383 2002/657/EC. The benzimidazole carbamate assay can screen for 11 residues at 50  $\mu\text{g kg}^{-1}$ ,  
384 equivalent to 25% of the concentration of the lowest MRL for benzimidazole carbamates in  
385 liver tissue. The amino-benzimidazole assay can screen for four benzimidazole residues at  
386 75  $\mu\text{g kg}^{-1}$ , which is 38% of the lowest MRL for amino-benzimidazoles in liver tissue. No  
387 false compliant results occurred during the study and the rate of false non-compliant  
388 samples was equal to 5% in both assays. Both screening assays can identify compliant  
389 liver tissue samples and thereby reduce the number of samples required to be tested by  
390 UHPLC/MS-MS. Only suspect non-compliant samples would then require confirmatory  
391 analysis by UHPLC-MS/MS. Using the methodology presented in this paper it is possible

392 to extract and analyse 25 samples within a single working day. This is the first reported  
393 immunochemical screening assay for amino-benzimidazole residues.

394

395

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### 402 References

403

- 404 [1] P. Delatour, G. Lorgue, D. Courtot, M. Lampras, *Bull. Soc. Sci. Vet. Med. Comp.* 77  
405 (1975) 197-203.
- 406 [2] M. Danaher, A.M. Sherry, J. O'Mahony, National Food Residue Database Report 2009.  
407 Teagasc, Dublin, 2009.
- 408 [3] A.M. Marti, A.E. Mooser, H. Koch, *J. Chromatogr. A* 498 (1990) 145-157.
- 409 [4] R.T. Wilson, J.M. Groneck, A.C. Henry, L.D. Rowe, *J. AOAC Int.* 74 (1991) 56-57.
- 410 [5] G. Domany, L. Koviacs, in: L.A. van Ginkel, A. Ruiters (Eds.), *Proceedings of the*  
411 *Euroresidue IV Conference on Residues of Veterinary Drugs in Food*, Veldhoven, The  
412 Netherlands, May 8-10, National Institute of Public Health and the Environment (RIVM),  
413 2000, pp. 361-370.
- 414 [6] G. Dowling, H. Cantwell, M. O'Keefe, M.R. Smyth, *Anal. Chim. Acta* 529 (2005)  
415 285-292.
- 416 [7] B. Kinsella, S.J. Lehotay, K. Mastovska, A.R. Lightfield, A. Furey, M. Danaher, *Anal.*  
417 *Chim. Acta* 637 (2009) 196-207.
- 418 [8] P. Delatour, R. Parish, in: A.G. Rico (Ed.), *Drug Residues in Animals*, Academic Press  
419 Inc., Orlando, FL, 1989, pp. 175-204.
- 420 [9] P. Delatour, F. Garnier, E. Benoit, C. Longin. *J. Vet. Pharmacol. Ther.* 7 (1984) 139-  
421 145.
- 422 [10] Q.A. McKellar, E.W. Scott, *J. Vet. Pharmacol. Ther.* 13 (1990) 223-247.
- 423 [11] D.L. Brandon, R.G. Binder, A.H. Bates, W.C. Montague Jr., *J. Agric. Food Chem.* 42  
424 (1994) 1588-1594.

425 [12] A-C, Huet, T. Fodey, S.A. Haughey, S. Weigel, C. Elliott, P. Delahaut., TrAC 29  
426 (2010) 1281-1294.  
427 [13] L. Johnsson, G.A. Baxter, S.R.H. Crooks, D.L. Brandon C.T. Elliott, Food Agric.  
428 Immunol. 14 (2002) 209-21 6.  
429 [14] J. Samsonova, G.A. Baxter, S.R.H. Crooks, A.E. Small, C.T. Elliott, Biosens.  
430 Bioelectron. 17 (2002) 523-520.  
431 [15] S.R.H. Crooks, B. McCarney, I.M. Traynor, C.S. Thompson, S. Floyd, C.T. Elliott,  
432 Anal. Chim. Acta, 483 (2003) 181-186.  
433 [16] S.R.H. Crooks, G.A. Baxter, M.C. O'Connor, C.T. Elliott, Analyst 123 (1998) 2755.  
434 [17] C. Situ, M.H. Mooney, C.T. Elliott, J. Buijs. TrAC 29 (2010) 1305-1315.  
435 [18] J. Keegan, M. Whelan, M. Danaher, S. Crooks, R. Sayers, A. Anastasio, C. Elliott, D.  
436 Brandon, A. Furey, R. O'Kennedy, Anal. Chim. Acta 654 (2009) 111-119.  
437 [19] European Commission Decision 2002/657/EC, Off. J. Eur. Comm. L221 (2002) 1-32.  
438 [20] B. Kinsella, M. Whelan, H. Cantwell, M. McCormack, A. Furey, S.J. Lehotay, M.  
439 Danaher, Talanta 83 (2010) 14-24.  
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446 **Table 1** Maximum residue limits (MRLs) for benzimidazole residues in liver.

447

448 **Table 2** Cross-reactivity profile of polyclonal amino-benzimidazole antibody (PAS 9869)  
449 polyclonal carboxy-albendazole antibody (S48) in HBS-EP buffer and ovine liver extract.

450

451 **Table 3** Determination of detection capability ( $CC\beta$ ) and repeatability of biosensor assays:  
452 Results from the analysis of fortified ovine liver ( $n = 20$ ) and the percentage recovery on  
453 different days ( $n = 5$ ).

454

455 **Table 4** Comparison between biosensor and UHPLC-MS/MS analysis of liver samples  
456 containing incurred mebendazole, fenbendazole and albendazole residues.

457

458

459 **Fig. 1.** Comparison of the sensitivity of different extraction methods for ABZ-SO analysis  
460 in ovine liver against equivalent curves in HBS-EP buffer.

461

462 **Fig. 2.** Calibration curves for 11 benzimidazole carbamates in ovine liver matrix.

463

464 **Fig. 3** Calibration curves for amino-benzimidazole metabolites in ovine liver matrix.

465

466 **Fig. 4.** SPR Biosensor assay calibration curves in fortified ovine liver on different days ( $n$   
467 = 5) for (A) albendazole sulphone (ABZ-SO<sub>2</sub>) and (B) albendazole-amino-sulphone (ABZ-

468 NH<sub>2</sub>-SO<sub>2</sub>).

Drug	Marker residue (possible metabolites)	MRL ( $\mu\text{gkg}^{-1}$ )	Animal species
Albendazole, Albendazole sulphoxide Netobimin	Sum of albendazole sulphoxide, albendazole sulphone, and albendazole 2-amino sulphone, expressed as albendazole	1000	All ruminants
Fenbendazole Febantel Fenbendazole-sulphoxide	Sum of extractable residues which maybe oxidised to fenbendazole sulphone	500	All ruminants
Thiabendazole	Sum of thiabendazole and 5-hydroxythiabendazole	400	Caprine
Flubendazole	Sum of flubendazole and amino flubendazole	400	Avian and porcine
Oxibendazole	Oxibendazole	200	Porcine
Triclabendazole	Sum of extractable residues that may be oxidised to keto-triclabendazole	250	All ruminants
Mebendazole	Sum of mebendazole, amino-mebendazole and hydroxymebendazole, expressed as mebendazole equivalents	400	Ovine, Caprine and Equidae

Table 2

Analyte	Amino-benzimidazole assay			
	Buffer		Liver	
	<sup>a</sup> IC <sub>50</sub> (ng mL <sup>-1</sup> )	<sup>b</sup> CR <sub>50</sub> (%)	<sup>c</sup> IC <sub>50</sub> (µg kg <sup>-1</sup> )	<sup>d</sup> CR <sub>50</sub> (%)
ABZ-NH <sub>2</sub> -SO <sub>2</sub>	5.7	100	44	100
FLU-NH <sub>2</sub>	7.1	80	55	80
MBZ-NH <sub>2</sub>	5.6	102	39	113
OXI-NH <sub>2</sub>	4.5	125	35	126
Analyte	Benzimidazole carbamate assay			
	<sup>a</sup> IC <sub>50</sub> (ng mL <sup>-1</sup> )	<sup>e</sup> CR <sub>50</sub> (%)	<sup>c</sup> IC <sub>50</sub> (µg kg <sup>-1</sup> )	<sup>d</sup> CR <sub>50</sub> (%)
ABZ	4.5	98	90	96
ABZ-SO	4.4	100	86	100
ABZ-SO <sub>2</sub>	4.8	93	87	99
FBZ	6.6	67	95	91
FBZ-SO	4.0	110	78	110
FBZ-SO <sub>2</sub>	4.0	110	82	105
MBZ	4.5	98	88	98
MBZ-OH	5.0	88	93	92
FLU	5.5	80	90	96
FLU-OH	6.6	67	89	97
OXI	6.2	71	88	98

<sup>a</sup> The concentration of analyte required to reduce the response by 50% in HBS-EP buffer.

<sup>b</sup> Cross-reactivity of antibody towards test amino-benzimidazole at 50% inhibition ( $(IC_{50}ABZ-NH_2-SO_2/IC_{50} \text{ test amino-benzimidazole}) \times 100$ ) in HBS-EP buffer.

<sup>c</sup> The concentration of analyte required to reduce the response by 50% in ovine liver.

<sup>d</sup> Cross-reactivity of antibody towards test amino-benzimidazole at 50% inhibition ( $(IC_{50} ABZ-NH_2-SO_2/IC_{50} \text{ test amino-benzimidazole}) \times 100$ ) in ovine liver.

<sup>e</sup> Cross-reactivity of antibody towards test benzimidazole at 50% inhibition ( $(IC_{50} ABZSO/IC_{50} \text{ test benzimidazole}) \times 100$ ) in HBS-EP buffer.

<sup>f</sup> Cross-reactivity of antibody towards test benzimidazole at 50% inhibition ( $(IC_{50} ABZSO/IC_{50} \text{ test BZT}) \times 100$ ) in ovine liver.

Table 3

Analyte	Assay Repeatability		Detection Capability	
	Mean recovery (%)	CV (%)	Mean $\pm$ S (n = 20)	CC $\beta$
	$\pm$ S (n = 5)	(n = 5)	( $\mu\text{g kg}^{-1}$ )	( $\mu\text{g kg}^{-1}$ )
	Fortification = 100 $\mu\text{g kg}^{-1}$		Fortification = 50 $\mu\text{g kg}^{-1}$	
ABZ	94 $\pm$ 11	11	66 $\pm$ 9	<50
ABZ-SO	105 $\pm$ 15	15	76 $\pm$ 9	<50
ABZ-SO <sub>2</sub>	122 $\pm$ 16	13	71 $\pm$ 5	<50
FBZ	132 $\pm$ 15	11	79 $\pm$ 8	<50
FBZ-SO <sub>2</sub>	127 $\pm$ 15	12	100 $\pm$ 15	<50
OFZ	113 $\pm$ 18	17	70 $\pm$ 7	<50
FLU	95 $\pm$ 13	13	55 $\pm$ 6	<50
FLU-OH	90 $\pm$ 9	10	59 $\pm$ 8	<50
MBZ	80 $\pm$ 11	13	51 $\pm$ 9	<50
MBZ-OH	77 $\pm$ 9	11	48 $\pm$ 11	50
OXI	106 $\pm$ 18	17	67 $\pm$ 9	<50
	Fortification = 125 $\mu\text{g kg}^{-1}$		Fortification = 75 $\mu\text{g kg}^{-1}$	
ABZ-NH <sub>2</sub> -SO <sub>2</sub>	109 $\pm$ 8	8	121 $\pm$ 26	<75
FLU-NH <sub>2</sub>	110 $\pm$ 18	16	87 $\pm$ 18	75
MBZ-NH <sub>2</sub>	116 $\pm$ 11	10	93 $\pm$ 14	<75
OXI-NH <sub>2</sub>	103 $\pm$ 9	9	105 $\pm$ 19	<75

Table 4

Sample	Species	Biosensor assays			UPLC-MS/MS assay		
		Benzimidazole carbamates ( $\mu\text{g kg}^{-1}$ )	Amino-benzimidazoles ( $\mu\text{g kg}^{-1}$ )	Interpretation	Concentration ( $\mu\text{g kg}^{-1}$ )	Analyte group	Status
1	Bovine	14	ND	Negative	ND	ND	C
2	Ovine	34	ND	Positive	13	FBZ	C
3	Ovine	19	ND	Negative	7	FBZ	C
4	Ovine	12	ND	Negative	5	FBZ	C
5	Ovine	60	ND	Positive	92	FBZ	C
6	Ovine	70	ND	Positive	75	FBZ	C
7	Ovine	>1000	ND	Positive	2659	FBZ	NC
8	Bovine	98	198	Positive	327	MBZ	NC
9	Bovine	>1000	ND	Positive	13096	FBZ	NC
10	Bovine	>1000	211	Positive	1222	ABZ	NC

<sup>a</sup>Negative samples = < CC $\beta$  and positive samples = > CC $\beta$ : where benzimidazole carbamate CC $\beta$  = 50  $\mu\text{g kg}^{-1}$  and amino-benzimidazole CC $\beta$  = 75  $\mu\text{g kg}^{-1}$

<sup>b</sup>UPLC-MS/MS concentrations are expressed as the sum of the FBZ, FBZ-SO and FBZ-SO<sub>2</sub> residues expressed as FBZ-SO<sub>2</sub>, MBZ, MBZ-NH<sub>2</sub> and MBZ-OH residues expressed as MBZ and ABZ, ABZ, ABZ-SO, ABZ-SO<sub>2</sub> and ABZ-NH<sub>2</sub>-SO<sub>2</sub> residues expressed as ABZ.

<sup>c</sup>C = compliant (< MRL) and NC = non-compliant (> MRL).

Figure 1B

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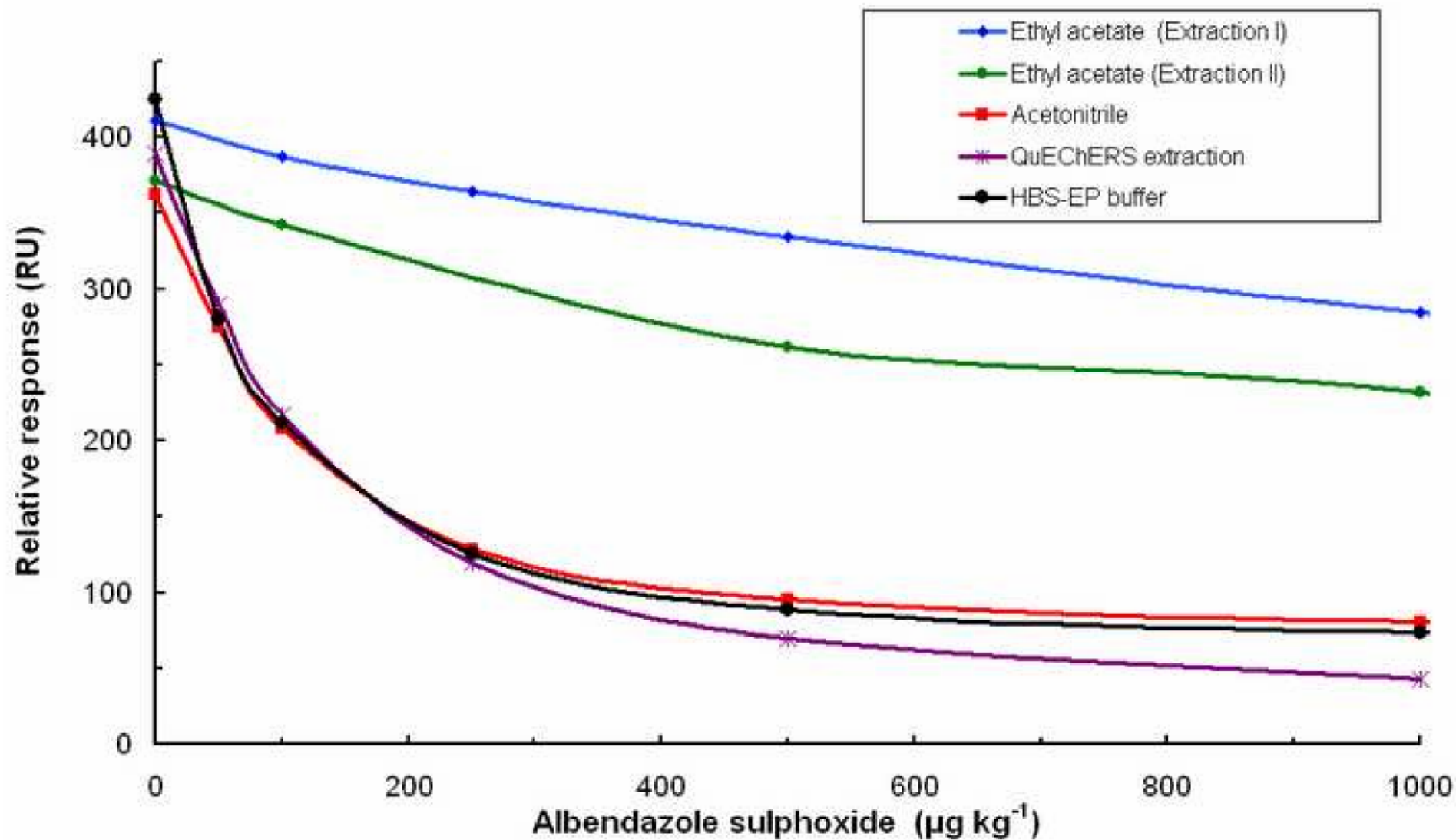




Figure 3B

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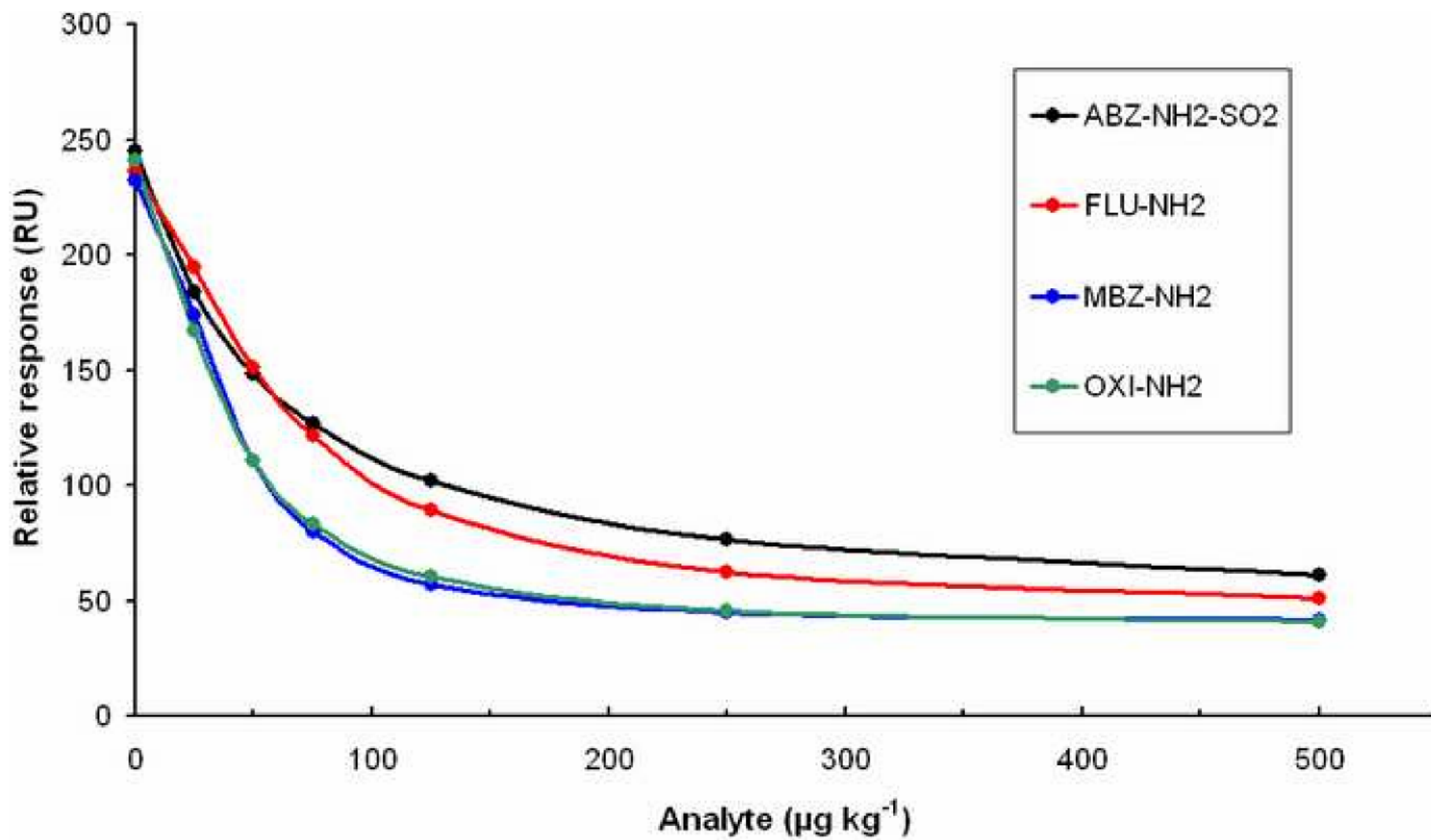


Figure 4B

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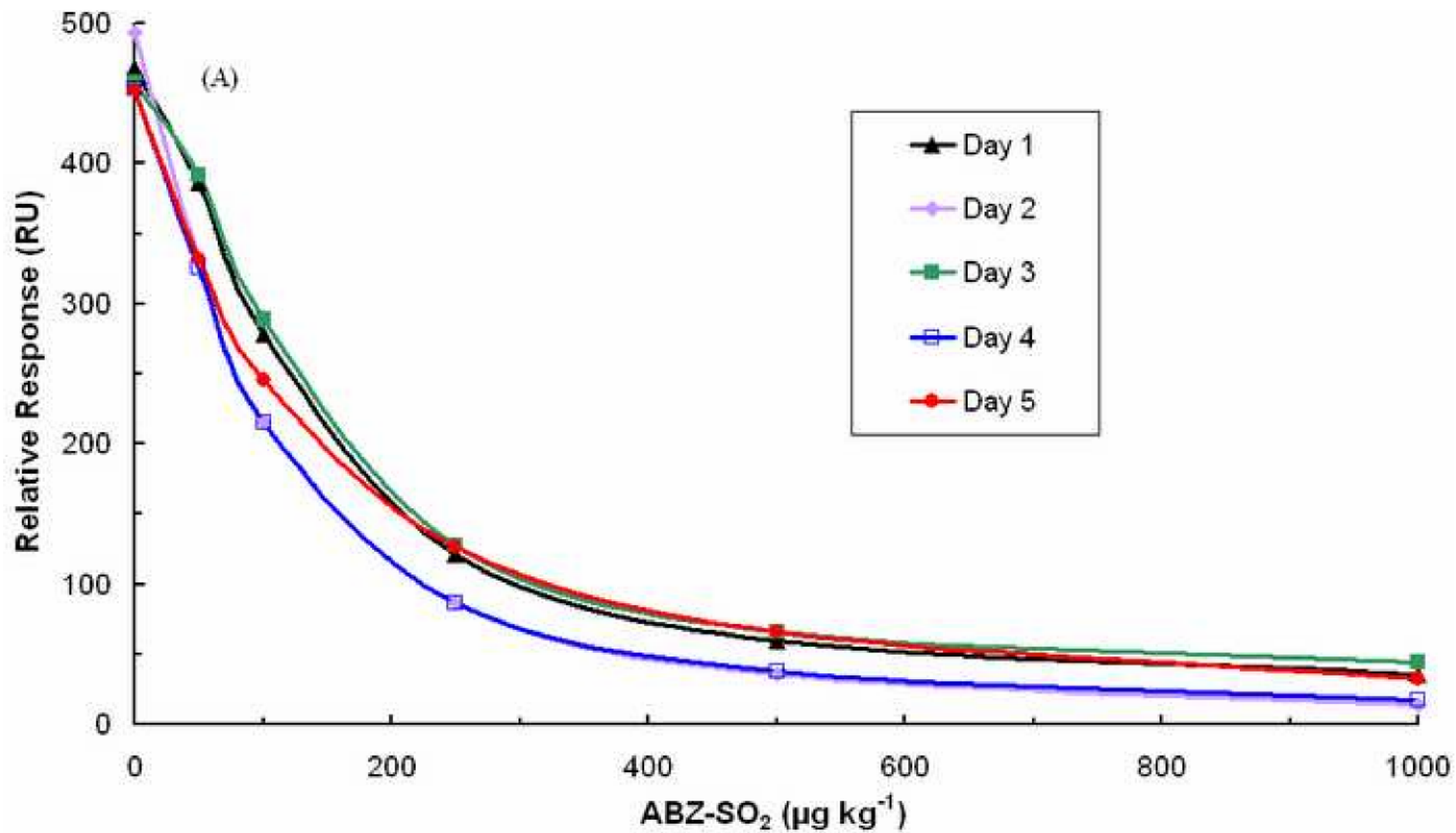


Figure 4B

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